2013

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J. Med. Entomol. 50(5): 1025–1030 (2013); DOI: http://dx.doi.org/10.1603/ME12175

ABSTRACT The stable fly, *Stomoxys calcitrans* (L.), is a cosmopolitan pest of livestock and humans. The pestiferous nature and painful bite cause stress to cattle and other animals. The stress and resulting avoidance behaviors manifest as reductions in weight gain or milk production in cattle; estimated annual economic loss in the United States exceeds US$2 billion. Understanding the population genetics of stable flies could provide information on their population dynamics, origins of outbreaks, and geographical patterns of insecticide resistance, resulting in a tactical advantage for developing management strategies. Previous studies, mostly on a local scale, reported a high level of gene flow between locations. Here, we report results wherein amplified fragment length polymorphism was used to determine genetic diversity of stable fly samples consisting of 11–40 individuals from 12 locations representing the United States, Canada, and Panama. The Analysis of Molecular Variance showed that the majority of genetic diversity was within groups; very little was among groups. The F_{ST} and G_{ST} values were low (<0.4), Nm values high (>1.0). The tests of neutrality suggested population expansion, and no genetic differentiation was found between locations. These results show that stable flies have a high level of gene flow on a continental scale, with limited isolation owing to distance or geographical barriers.

KEY WORDS *Stomoxys calcitrans*, population genetics, AFLP, veterinary insect pest

Stomoxys calcitrans (L.) is a hematophagous pest with a global distribution. In North America, it is the primary pest of cattle, causing >US$2 billion in economic losses annually (Taylor et al. 2012). Therefore, the majority of research on this pest has concentrated on its control in livestock facilities (Kneeland et al. 2012b). Successful stable fly population control and reduction relies on integrated pest management (IPM) techniques implementing a high level of sanitation and well-timed insecticide applications, yet consistent reduction of stable fly numbers may not result (Campbell 1995), perhaps because of their dispersal capacity (Berkebile and Thomas 1995). Resistance to certain insecticides has also been reported in stable flies (Cilek and Greene 1994, Pitzer et al. 2010). Elucidating the genetic variability of stable flies may be helpful both in determining sources from which stable flies disperse and for monitoring insecticide resistance.

Several methods have been developed to more efficiently analyze insect genomes. The most frequently used methods have been microsatellite analysis and direct DNA sequencing of mitochondrial genomes (Gilles et al. 2004, Marquez et al. 2007, Dsouli-Aymes et al. 2011). Although plant scientists have been using amplified fragment length polymorphism-polymerase chain reaction (AFLP-PCR) (Bensch and Åkesson 2005), the technique has only recently been adopted for population genetic analyses of insects. For example, AFLP markers have been used to study the genetic structure of the fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Martinelli et al. 2007), in Brazil and the European corn borer, *Ostrinia nubilalis* (Hubner), in the midwestern United States (Krumm et al. 2008). Recently, Lindroth et al. (2012) studied the genetic variation of western bean cutworm, *Striacosta albicosta* (Smith), across the United States, and Kneeland et al. (2012a) evaluated the genetic variation among laboratory populations and field populations of the spined soldier bug, *Podisus maculiventris* (Say), in Missouri.

AFLP is an attractive technique for conducting population genetic analyses because it is cost effective, generates a large number of dominant markers, which can be scored using computer software, is more reproducible than Random Amplified Polymorphic DNA, and requires only a minute amount of genomic

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Table 1. Collection sites, including number of samples, geographical coordinates, and elevation

Location, city, state	Coordinates	Elevation
Potrerillos Arriba, Dolega, Chiriqui, Panama (40)	8.410396 N, 82.290556 W	869.3 m (2,852 ft)
Lethbridge, Alberta (40)	49.413656 N, 112.503062 W	893.0 m (2,930 ft)
Russell, Ontario (40)	45.152633 N, 75.213009 W	68.9 m (226 ft)
Jonesboro, Arkansas—ASU Farm (24)	35.502237 N, 90. 421540 W	97.2 m (319 ft)
Jasper Co., Indiana (38)	39.031979 N, 88.052427 W	165.8 m (544 ft)
Manhattan, Kansas (40)	39.110099 N, 96.341801 W	361.2 m (1,185 ft)
Montana—Medicine Lake NWR (39)	47.585650 N, 107.563472 W	865.0 m (2,838 ft)
Lincoln, Nebraska (38)	40.495959 N, 96.393773 W	353.6 m (1,160 ft)
Raleigh, North Carolina—NCSU Dairy (25)	35.494691 N, 78.430072 W	109.7 m (360 ft)
Kerrville, Texas (23)	30.0428 N, 99.3854 W	536.8 m (1,761 ft)
Washington (eastern) (27)		
Frazier (Moxee)	46.528205 N, 120.374317 W	347.8 m (1,141 ft)
Ferguson (Moxee)	46.528205 N, 120.374317 W	347.8 m (1,141 ft)
Russell (Prosser)	46.133357 N, 119.442946 W	205.7 m (675 ft)
Stark	46.506087 N, 120.193906 W	449.3 m (1,474 ft)
Washington (western) (11)		
Carstens	48.4877 N, 121.703 W	211.2 m (693 ft
Silvana	48.241 N, 122.369 W	30.8 m (101 ft)

DNA (Bensch and Åkesson 2005). Herein, AFLP was used to investigate the genetic diversity of stable flies. The working hypothesis was that AFLP would reveal genetic differentiation across major geographical barriers. If accepted, this would support the results of Marquez et al. (2007) and Dsouli–Aymes et al. (2011), suggesting that there are isolated subpopulations of stable flies. Alternatively, high genetic variation between individuals but minimal genetic variation between collection areas would support the results of Jones et al. (1991) and Szalanski et al. (1996), and suggest that stable flies are a panmictic population within North America.

Materials and Methods

Samples. Samples were obtained by collaboration with colleagues in each location (Table 1). Owing to the diversity of locations and donors, different collection methods were used, including sweep netting and sticky traps; all samples were placed in 95% ethanol. Immediately on arrival, all vials or tubes containing samples were refreshed with 95% ethanol and stored at 4°C until DNA extraction. In most cases an abundance of samples were received. Some locations were unable to collect the requested 50 flies, but only one location (Western Washington, 11 samples) had <20 flies that were analyzed. When possible, 40 flies from each location were analyzed; sample size ranged from 11 to 40 (Table 1).

DNA Extraction. DNA was extracted using the CTAB (Hexadecyltrimethylammonium bromide) method modified from Doyle and Doyle (1987). Samples were washed in autoclaved double distilled water for >10 min. The head, abdomen, wings, and legs were detached from the thorax, and the gut was removed. The thorax was homogenized in 250 μl of CTAB buffer (100 mM Tris-HCl, 1.4 M NaCl, 0.02 M EDTA, 2% CTAB, 0.2% β-mercaptoethanol) in a 1.5-ml microcentrifuge tube, then an additional 250 μl CTAB was added for a total of 500 μl CTAB. A few grains of sea sand (Fisher, Pittsburg, PA) were added to facilitate homogenization. DNA extraction then proceeded following a protocol common to our laboratory, described in previous publications (Krumm et al. 2008, Tiroesele 2011, Lindroth et al. 2012).

After the DNA had been extracted, all samples were analyzed for DNA quality and quantity on the Nanodrop spectrophotometer (ThermoScientific, Wilmington, DE). The pedestal was cleaned with a kimwipe after each sample, and washed with autoclaved nanopure water after each group of 20 samples. After each location (40 samples), the pedestal was washed and the spectrophotometer was reblanked with 1× TE.

Amplified Fragment Length Polymorphism-Polymerase Chain Reaction. AFLP-PCR was performed using a protocol modified from Vos et al. (1995), commonly used in our laboratory (Alamalakala et al. 2009, Kneeland et al. 2012a).

Before proceeding with selective amplification, a primer test was performed to determine which primer pairs would work best for stable fly DNA. A matrix of the available primers was created, and primer pairs were tested using DNA from a previous project known to be of good quality. Based on the test, the primer sets chosen for this project were M-CAC and E-AAC, M-CTA and E-AAC, M-CTC and E-AAC, and M-CTC and E-ACA (Table 2). Optimization tests were performed to determine the correct amount of reagents to use to obtain the best results with stable flies. The resulting PCR mix consisted of 1.2 μl 10× PCR buffer, 0.72 μl $MgCl_2$, 0 0.07 μl Amplitaq 360 DNA polymerase (Applied Biosystems, Grand Island, NY), 0.4 μl 10 mM dNTP mix, 0.75 μl 5 μM M-primer, 0.3 μl 1 μM E-primer, and 6.79 μl dH_2O. Ten microliters of the selective mix were dispensed into 0.2 ml PCR tubes, and 2 μl of diluted template DNA was added to each tube. Two sample locations were prepared at one time. PCR conditions for samples were one cycle of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min; 12 cycles of 94°C for 30 s, 65°C for 30 s (with the 65°C annealing temperature decreasing by 0.7°C each cycle to a final tem-

Table 2. Nucleotide sequences of adapters, preamplification primers, and selective primers used

Primer ID	Primer type	Sequence
EcoRI-1 (forward)	Adapter	5-CTCGTAGACTGCGTACC-3
EcoRI-2 (reverse)	Adapter	5-AATTGGTACGCAGTCTAC-3
MseI-1 (forward)	Adapter	5-GACGATGAGTCCTGAG-3
MseI-2 (reverse)	Adapter	5-TACTCAGGACTCAT-3
E (N+0)	Preamp primer	5-GACTGCGTACCAATTC-3
M (N+1)	Preamp primer	5-GATGAGTCCTGAGTAAC-3
M-CAC	Selective primer	5-GATGAGTCCTGAGTAACAC-3
M-CTA	Selective primer	5-GATGAGTCCTGAGTAACTA-3
M-CTC	Selective primer	5-GATGAGTCCTGAGTAACTC-3
E-AAC	Selective primer	5-GACTGCGTACCAATTCAAC-3
E-ACA	Selective primer	5-GACTGCGTACCAATTCACA-3

Sequences were described by Vos et al. (1995). EcoRI selective primers (E-AAC and E-ACA) were tagged with fluorescent dye.

perature of 56°C), and 72°C for 1 min; 23 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min and a 4°C hold.

Following the selective amplification, samples were denatured by adding 2.5 μl blue stop solution (LICOR Biosciences, Lincoln, NE) to each tube and denaturing at 95°C for 3 min, then electrophoresed on 6.5% polyacrylamide gel.

Data Analysis. Gels were scored for presence or absence of bands using SAGA Generation two software (LICOR Biosciences, Lincoln, NE). After scoring, reports were generated as binary matrices and formatted for specific genetic analysis software. Programs used for analysis included Arlequin (Excoffier et al. 2005), PopGene32 (Yeh and Boyle 1997), and Bootsie (J.E.F., unpublished data). Additional AFLP-specific software programs were used for verification (data not shown). Analyses included the analysis of molecular variance (AMOVA), Nei's genetic diversity, mismatch distribution, Tajima'SD and Fu's F_S tests of neutrality, and percentage coefficient of variation. Geographic groupings (North–South and East–West) used for the AMOVA analysis and population dendrograms are indicated in Table 3.

Results

In total, 433 individual stable flies were analyzed from 12 locations in the Western Hemisphere, an average of 36 flies per location. The four primer pairs generated 387 AFLP markers per individual, with a total of 167,571 loci analyzed. Bootsie results indicate that 96% of the genetic variation can be accounted for with these 387 markers (data not shown). The majority of the markers were polymorphic, with frequencies >5%. There were very few locally common bands (Fig. 1).

Analysis of Molecular Variance and Nei's Genetic Diversity. The majority of the variation in the stable fly samples occurred within populations (71.00–89.92%), with 10.07–29.10% of the variation occurring among populations within groups (Table 3). A very low percentage (0.0–0.70%) of variation occurred among groups. Genetic diversity values using Wright's F-statistics (F_{ST}) (Wright 1950), Nei's coefficient of gene differentiation (G_{ST}) (Nei 1973), and the comparable values PhiPT and PhiST ranged from 0.128 to 0.382, suggesting a large amount of genetic variation yet some genetic differentiation. The Nm value (1.43), suggests that there is a high amount of gene flow between locations.

Tests of Neutrality. The results of the neutrality test show positive deviations from zero for D, but they were not significant. Fu's Fs are negative (significant at 0.02), suggesting population expansion (Fu 1997).

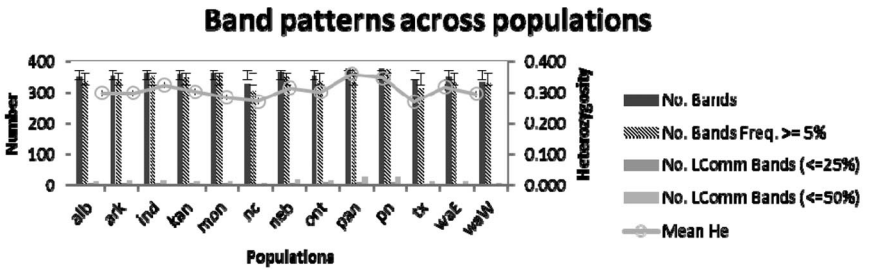
Table 3. AMOVA results from Arlequin, GenAlEx, and FAMD software, showing the percent variation among groups, among populations within groups, and within populations

Analysis of molecular variance			
Software program	Percent variation among groups	Percent variation among populations within groups	Percent variation within populations
Arlequin East–West[a]	−0.94	29.10	71.84
Arlequin North–South[a]	0.70	27.95	71.35
GenAlEx		29.00	71.00
FAMD		10.07	89.92

Location groups used for the analysis of molecular variance[b]

North–South				East–West			
Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
Alberta	Indiana	Arkansas	Panama	Alberta	Kansas	Arkansas	N. Carolina
Montana	Kansas	N. Carolina		Montana	Nebraska	Indiana	Panama
Ontario	Nebraska	Texas		Wash E	Texas	Ontario	
Wash E				Wash W			
Wash W							

[a] Results of North–South and East–West groupings were identical. GenAlEx and FAMD did not give among group results.
[b] Locations were grouped in a North–South transect and an East–West transect.



No. Bands = No. of different bands
No. Bands Freq. >=5% = No. of different bands with a frequency >= 5%
No. LComm Bands (<=25%) = No. of locally common bands (Freq. >=5%) found in 25% or fewer populations
No. LComm Bands (<=50%) = No. of locally common bands (Freq. >=5%) found in 50% or fewer populations

Fig. 1. Number of polymorphic loci and expected heterozygosity in the stable fly populations by location. The majority of the loci are polymorphic. No. Bands = Number of different bands. No. Bands Freq. ≥5% = Number of different bands with a frequency ≥5%. No. LComm Bands (≤25%) = Number of locally common bands (Freq. ≥5%) found in 25% or fewer populations. No. LComm Bands (≤50%) = Number of locally common bands (Freq. ≥5%) found in 50% or fewer populations.

Mismatch Distribution. None of the SSD values were significant although four populations were approaching significance. All samples had a very low raggedness index, which indicates population expansion.

Mantel Test. The Mantel test (Mantel 1967) compares a genetic distance matrix with a geographical distance matrix to test for correlation between genetics and geographical location. The genetic distance matrix was used as the X matrix and geographical distance was used for Y. The mean value of Y is the mean genetic differences between samples, and the mean value of X is the mean distance in kilometers between collection locations. The low regression coefficient R^2 (0.0068) and shotgun pattern of the graph indicate that there is no correlation between geographical location and genetic distance in these samples (Fig. 2).

Dendrogram. The unweighted pair-group method with arithmetic average method was used to construct the dendrogram (Fig. 3); this method assumes that evolutionary (mutational) rates are equal for each group (Michener and Sokal 1957). Outliers were used as a control to demonstrate the efficacy of the unweighted pair-group method with arithmetic average method of building the dendrograms, and were not included in data analysis. Grouping the populations into East–West and North–South transects did not alter the dendrogram.

Discussion

Studies of stable fly populations on a regional scale have reported a large amount of genetic variation and gene flow between locations (Szalanski et al. 1996, Gilles et al. 2007). Conversely, recent global studies have reported genetic differentiation and isolated populations (Marquez et al. 2007, Dsouli–Aymes et al. 2011). The results of our study on a continental scale indicate that major geographical barriers such as the Rocky Mountains, Appalachians, and Sierra Madres do not inhibit gene flow between stable fly populations, most likely owing to the transportation of livestock.

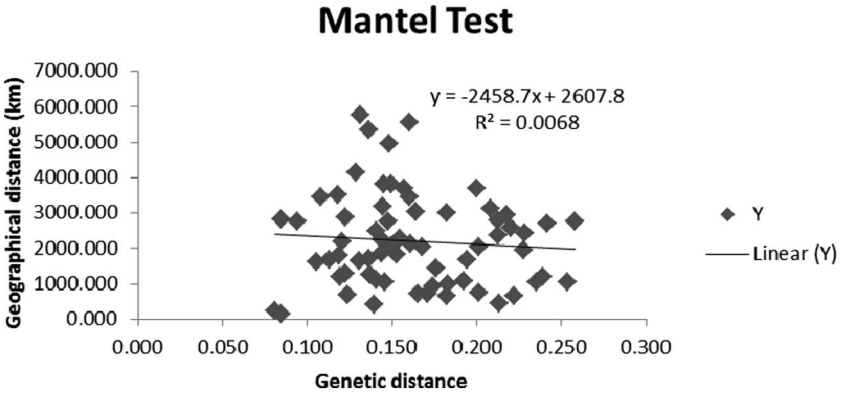


Fig. 2. Mantel test, based on the correlation between a genetic distance matrix and a geographical distance matrix. The Mantel test performs permutations on one matrix while holding the other constant. The regression coefficient R^2 is very low (0.0068), indicating that there is no correlation between genetic and geographic distance in the stable fly samples analyzed.

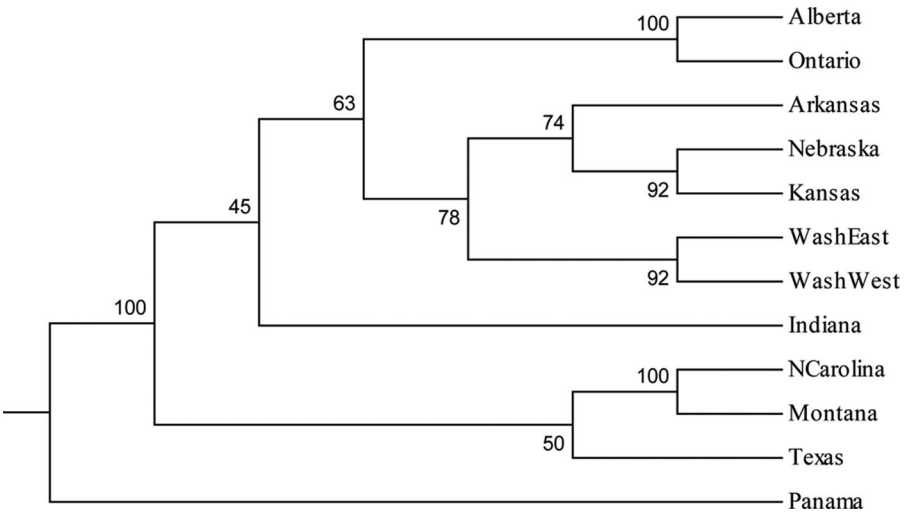


Fig. 3. Dendrogram generated using the Unweighted Pair Group Method with Arithmetic Mean, which assumes a constant evolutionary rate. The numbers represent bootstrap values.

In this study, the F_{ST} and G_{ST} values are consistently below 0.4, indicating some genetic differentiation, and the Nm was high (>1.00), indicating that migration could help maintain genetic variability. Possibly stable fly, as a recent introduction to North America, has not experienced sufficient time to genetically differentiate. Tajima's SD and Fu's Fs tests of neutrality and the mismatch distributions suggest that these populations may have experienced (or are experiencing) population expansion (data not shown). These results support the hypothesis that stable flies originated in one region and populations expanded over time (Zumpt 1973).

The regression coefficient, R^2, generated by the Mantel test is very low at 0.0068. This number and the scatter plot indicate that there is no correlation between genetic distance and geographical distance in these samples, again supporting nonsignificant genetic differentiation in North American stable flies.

The dendrogram suggests that there could have been several introductions into the New World from different areas. They would likely have been introduced from the Palearctic region, because of colonization of North America by the Europeans, along with possible introductions from Africa during slave-trading activities. The groupings in the dendrogram suggest multiple origins and a large amount of gene flow between New World areas. Considering that the results from the Arlequin analyses and Nei's genetic diversity were consistent throughout, there could be many explanations for the clustering of samples from divergent locations in the dendrogram. Human migration and colonization may have affected the expansion of the stable fly population. *S. calcitrans* is a synanthropic, generalist pest of livestock. There has been an influx of humans and their livestock into the New World from the Palearctic region. As the human population spread across the Americas, the stable fly population expanded as well, feeding on livestock and increasing in numbers. Now, with humans and stable flies occupying the entire continent, stable fly populations continue expanding because of the movement of livestock for economic and recreational activities. Considering the dynamic movement of humans and livestock, it is not unexpected that stable fly populations are dynamic, with a very high degree of gene flow across the New World.

This project revealed significant information on the potential origin of stable flies in the New World. It showed that geographical features such as mountain ranges may not be a barrier to gene flow, and there currently are no genetically isolated populations within the areas analyzed. This information is of benefit when managing pest populations, as pest management strategies should have a similar effect on stable flies in all locations. This project did not address the origin of stable flies in local areas, but some patterns arose in the dendrograms that may be useful for further research in that area. Alberta and Ontario, and Nebraska and Kansas, always group together in this study which could be explained by trade in the cattle industry between the midwestern United States and Canada.

Future research, with larger sample sizes in locations such as eastern and western Washington, could be analyzed to clarify the genetic diversity of stable flies on this local scale. Larger-scale global projects would increase the current knowledge of global population structure. As recommend in Bensch and Åkesson (2005), we support and recommend using AFLP because of its low start-up time and cost-effective generation of data from a large number of evenly distributed loci in the whole genome. Providing insight into stable fly population dynamics both on the local scale and globally will ultimately contribute to future pest management strategies.

Acknowledgments

Many thanks to the following individuals for supplying stable flies for this project: Dennis Berkebile (U.S. Department of Agriculture–Agriculture Research Services, Lincoln, NE); Holly Ferguson (WSU Extension, IAREC, Prosser, WA); Gregory Johnson and Kristina Hale (Montana State University, Bozeman, MT); Wes Watson (North Carolina State University, Raleigh, NC); Ludek Zurek (Kansas State University, Manhattan, KS); Tanja McKay (Arkansas State University, State University, AR); Kevin Floate (Agriculture and Agri-Food Canada, Lethbridge, Alberta); Steven Mihok (Russell, Ontario); Ralph E. Williams (Purdue University, West Lafayette, IN).

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Received 7 August 2012; accepted 28 May 2013.